



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
PATENT  
Atty. Dkt. No. 6208.570

BOX Patent Application  
Commissioner of Patents and Trademarks  
Washington, DC 20231

Sir:

Transmitted herewith for filing is the patent application of:

Inventor(s): Tsukasa Matsumoto

For: METHOD FOR FRACTIONING RED BLOOD CELLS AND ANTIBACTERIAL MATERIALS  
OR BACTERIAL PROLIFERATION INHIBITORS PRODUCED THEREBY

Enclosed are:

- Specification, claims and abstract, 15 total pages
- 1 sheets of ~~xx formal~~/formal drawings
- An Assignment of the invention to \_\_\_\_\_
- Executed Declaration and Power of Attorney
- A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27
- Preliminary Amendment
- Information Disclosure Statement and Form PTO-1449 w/references cited
- Certified copy of \_\_\_\_\_ Application No. \_\_\_\_\_ from which priority is claimed is attached/will follow.

The filing fee has been calculated as shown below:

CLAIMS FOR FEE CALCULATION

	Number Filed	Number Extra	Rate	Basic Fee \$ 770.00
Total Claims	9	-20 = 0	x \$ 22.00	0
Independent Claims	9	-3 = 6	x \$ 80.00	480.00
Multiple dependent claim(s), if any		0	\$ 260.00	0
Filing Fee Calculation				\$ 1,250.00
SMALL ENTITY FILING FEE CALCULATION (50% OF ABOVE)				\$ 625.00

- A check in the amount of \$ 625.00 is attached for the filing fee and \_\_\_\_\_.
- Please charge Deposit Account No. 13-5132 in the amount of \$ \_\_\_\_\_ for the filing fee and \_\_\_\_\_.
- The Commissioner is hereby authorized to charge any additional fees which may be required by this paper or credit any overpayment to Account No. 13-5132:
  - 37 CFR 1.16 (filing fees)
  - 37 CFR 1.16 (presentation of extra claims)

A duplicate copy of this sheet is enclosed.

Joseph W. Berenato, III, Esq.  
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Docket No.: 6208.570

Tsukasa Matsumoto

Applicant  
For:  
Patent & Trademark Office

METHOD FOR FRACTIONING RED BLOOD CELLS AND ANTIBACTERIAL MATERIALS OR BACTERIAL PROLIFERATION INHIBITORS PRODUCED THEREBY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
-- (37 CFR 1.9(f) and 1.27(b)) - Independent Inventor

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled

METHOD FOR FRACTIONATING RED BLOOD CELLS AND ANTIBACTERIAL MATERIALS OR BACTERIAL PROLIFERATION INHIBITORS PRODUCED THEREBY

described in the specification filed

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

I acknowledge the duty to file, in this application or patent, notification to any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fees due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Tsukasa MATSUMOTO

Name of Inventor

Name of Inventor

Name of Inventor

*Tsukasa Matsumoto*

Signature of Inventor

Signature of Inventor

Signature of Inv.

January 20, 1997

Date

Date

Date



625-201

59227 U.S. PTO  
08803458  
02/20/97

A

## SPECIFICATION

### TITLE OF THE INVENTION

5       METHOD FOR FRACTIONATING RED BLOOD CELLS AND ANTIBACTERIAL  
MATERIALS OR BACTERIAL PROLIFERATION INHIBITORS  
PRODUCED THEREBY

### BACKGROUND OF THE INVENTION

10

#### 1. Field of the Invention

The present invention relates to methods for fractionating red blood cells (RBC) of human blood into several fractions having different functions. Further the present invention 15 relates to specific materials produced by such method, which are possessed of antibacterial properties or inhibitory against bacterial proliferation.

#### 2. Prior Art

20       Although it has been conventionally known that red blood cells of human blood act as carriers for carrying a large amount of oxygen and carbon dioxide at a high speed, the other functions of red blood cells have not yet been known completely.

25

### SUMMARY OF THE INVENTION



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It is therefore a primary object of this invention to find new functions of red blood cells which are greater parts of human blood.

5 It is still more specific object of this invention to provide methods for fractionating red blood cells into different functions, which method can be used for such research.

In order to achieve the above objects, the inventor of the present invention has been performed various researches for fractionating red blood cells of human blood into several 10 fractions. The inventor found that the tested blood could be fractionated into three layers after the blood was added with dextran and then maintained under a certain condition for a certain period. The fractionated blood cells contained these 15 three layers provided different functions on bacteria, respectively. The present invention is based on this knowledge.

The above described objects are accomplished by the method for fractionating red blood cells of human blood into three fractions according to the present invention which comprises following steps; (a)human blood sample is mixed with dextran 20 aqueous solution and maintained stationarily for 60 to 75 min to fractionate this blood sample into three layers; (b)these upper, intermediate, and lower layers are individually separated and collected; and (c)the upper layer sample is treated with hypotonic solution for a short period and then 25 added with hypertonic solution.

Further, the present invention may provide another method for producing a fraction including antibacterial red blood

cells, which comprises following steps; (a)human blood sample is mixed with dextran aqueous solution and maintained stationarily for 60 to 75 min to fractionate this blood sample into three layers; (b)the upper layer is separated from the other layers; and (c)the upper layer is treated with hypotonic solution for a short period and then added with hypertonic solution.

Furthermore, the present invention may provide a still other method for producing a fraction including bacterial proliferation inhibitory red blood cell, which comprises following steps; (a)human blood sample is mixed with dextran aqueous solution and maintained stationarily for 60 to 75 min to fractionate this blood sample into three layers; and (b)the intermediate layer sample is separated and collected from the other layers.

Accordingly, the methods provided by the present invention can easily fractionate three different blood fractions including red blood cells as a main component and having different functions. These three different fractions may be applied to several clinical tests such as antibacterial test and the like.

These and other objects and many of the attendant advantages of this invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawing.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 is a diagrammatic illustration of changes in the shape of red blood cells included in the fractions provided by  
5 the methods according to the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

First, according to one preferred embodiment of the present  
10 invention, human blood (including all blood ingredients) is mixed with dextran aqueous solution. This dextran aqueous solution used in the present invention is preferably selected from 5 to 10 w/v% of dextran aqueous solution or physiological saline solution, more preferably 5 to 10 w/v% of dextran  
15 physiological saline solution, most preferably 5 to 8 w/v% of dextran aqueous solution to be added into the blood sample is preferably one to twice the volume of the blood sample, more preferably 1.5 times. The mixture of the blood and the dextran aqueous solution is sufficiently stirred and then remained stationarily  
20 for 60 to 75 minutes. The mixture becomes forming three layers (upper, intermediate, and lower layers).

The upper layer is separated from the other layers by means of a pipette. The separated layer is treated with hypotonic solution for a short period and then added with hypertonic solution. Finally, one fraction containing red blood cells providing antibacterial activity is produced. In practical  
25

manner, the upper layer is subjected to centrifugal separation to collect blood cells. The separated blood cell sample is further added with hypotonic solution, and after a short period, added with hypertonic solution to provide isotonic solution. In the present invention, a saponin solution is preferably used for the hypotonic solution, but any materials capable of making the hypotonic solution to isotonic may be used for the hypertonic solution. Such short period for remaining the blood cell sample in the hypotonic solution is preferably about 30 seconds. If the treatment period in the hypotonic solution is shorter, the produced sample will not be antibacterial. On the other hand, if longer, hemolysis will excessively progress and thus the sample includes less red blood cells.

The intermediate and lower layers may be selectively taken from the separated layers by means of a pipette, respectively.

Such prepared three red blood cell fractions are usually added into liquid medium such as RPMI-1640, MEM, BME, Ham F12, MCDB104, MCDB153, and so on to be applied to various researches and studies such as observation of act on various bacteria.

One experiment wherein specific bacteria were inoculated upon the above hypotonic treated upper layer indicated that the red blood cells contained in the upper layer attacked upon these bacteria while the leucocytes contained in the upper layer did not act on these bacteria at all. In other words, the red blood cells contained in the upper layer provide antibacterial function. When the same bacteria were inoculated



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upon the intermediate layer, this experiment resulted in that proliferation of bacteria was remarkably inhibited. Also when the lower layer was subjected to the similar experiment, this test resulted in no action on the inoculated bacteria.

5 According to another preferred embodiment of the present invention, the same three red blood cell fractions; the upper, intermediate and lower layers, were fractionated and prepared as liquid medium in the same manner as above. Incubated leucocyte sample taken from another human blood was added into  
10 the upper and intermediate layers, respectively. In order to observe activation on bacteria in these fractionated samples, specific bacteria were immediately added into the upper and intermediate layers including the incubated leucocytes, respectively. These samples were observed three times at  
15 intervals of 24 hours; i.e., after 24, 48, 72 hours. These observations resulted in that proliferation of bacteria was remarkably inhibited. Reference tests using commercial available antibiotics were carried out on the same occasion to compare with the above results according to the present  
20 invention. This comparison also proved that the upper and intermediate layer samples provided superior inhibitory on bacteria rather than all antibiotics.

As is clear from these experimental tests, specific materials having stronger inhibitory effect on proliferation 25 of bacteria rather than any existing antibiotics are generated in the liquid medium samples composed of the upper layer including red blood cells and incubated leucocytes added

thereto, and the intermediate layer including red blood cells and incubated leucocytes added thereto, and further their activated condition is maintained stably for a long period.

It should be understood that such inhibitory materials are either cell-products composed of the red blood cells contained in each layer and the leucocytes added thereto, or by-products secreted out of the blood cells. It should be understood that such by-products are the first type materials secreted out of the red blood cells in the upper or intermediate layer acted by the incubated leucocytes added thereto; the second type materials generated when the incubated leucocytes are broken or secreted out of the incubated leucocytes; or the third type materials generated by the cooperation between the red blood cells of each fraction and the incubated leucocytes. In order to specify such inhibitory materials, the cultured solution of the red blood cell fraction added with the incubated leucocytes is subjected to analysis via filtering and extracting processes.

The present invention will be further understood by the following example. However, the present invention is not limited to this example.

#### Example

Fresh human blood 10 ml of a healthy person was added with dextran solution 15 ml (which was prepared by dissolving 7.0 g of Dextran 70 manufactured by Tokyo Kasei Co., Ltd in 100 ml of physiological saline solution), and sufficiently stirred by a

pipette, and then maintained stationarily for 60 to 75 min. This sample solution was separated into three layers, the upper, intermediate, and lower layers.

The upper layer was subjected to a centrifugal separation  
5 to precipitate blood cells (red blood cells and leucocytes). This precipitated part was added with hypotonic solution (RLB, produced by Harajuku Clinic) 30 ml, and after 30 sec, added with hypertonic solution (BELMAR, produced by Harajuku Clinic) 10 ml. This mixture was subjected to a centrifugal separation to  
10 collect blood cells. Thus collected blood cells were further added with liquid medium (RPMI-1640) 3 ml. This solution was referred to "sample A".

Four drops of the intermediate layer were taken up by a pipet. This drop-solution was added with liquid medium  
15 (RPMI-1640) 3 ml. This solution was referred to "sample B".

A drop of the lower layer was taken up by a pipet. This drop-solution was added with liquid medium (RPMI-1640) 3 ml. This solution was referred to "sample C".

## 20 Test and Results

A small amount of *Pseudomonas aeruginosa* was inoculated upon the samples A, B, C and RPMI-1640 (as a control sample), respectively. These samples were remained for 5 to 6 hours at room temperature, and then incubated under the condition of 5%  
25 CO<sub>2</sub>, 37°C. Thus resulted samples A, B, C and control sample were observed through a microscope to know the action of the samples A, B, C and control sample on the inoculated bacteria.

The microscopic observation resulted in that the sample A included some leucocytes in addition to red blood cells, and the red blood cells were changed into various shapes as shown in Fig. 1. According to the observation of the sample A  
5 incubated for six hours, the leucocytes did not positively act on the inoculated bacteria at all, while the red blood cells changed in their shapes moved toward the inoculated bacteria and thus the bacteria were prohibited from moving freely.

The microscopic observation resulted in that the sample B  
10 also included a small amount of leucocytes in addition to red blood cells, and the red blood cells were changed into various shapes as shown in Fig. 1. According to the observation of the sample B incubated for six hours, the inoculated bacteria were remarkably decreased in comparison with the control sample.

15 According to the microscopic observation of the sample C, the sample C included only red blood cells which were changed into various shapes. The counted number of the inoculated bacteria in the sample C were substantially equal to that of the control sample.

20 As is clear from the above description, the methods provided by the present invention can easily fractionate three different blood fractions including red blood cells as a main component and having different functions. These three different fractions may be applied to several clinical tests such as  
25 antibacterial test and the like.

As many apparently widely different embodiments of this invention may be made without departing from the spirit and

**scope thereof, it is to be understood that the invention is not limited to the specific embodiments thereof except as defined in the appended claims.**

JULY 22 1998 33360

WHAT IS CLAIMED IS:

1. Method for fractionating red blood cells of human blood  
5 into three fractions comprising following steps;

(a)mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

10 (b)separating said three layers into three individual samples; and

(c)treating the upper layer sample with hypotonic solution for a short period and then adding hypertonic solution into said upper layer sample.

15 2. Method for producing a fraction including antibacterial red blood cells, which comprises following steps;

(a)mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

20 (b)separating and collecting the upper layer from the other layers; and

(c)treating the upper layer sample with hypotonic solution for a short period and then adding hypertonic solution into said upper layer sample.

25 3. Method for producing a fraction including bacterial proliferation inhibitory red blood cells comprising following

steps;

(a)mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers; and

(b)separating and collecting the intermediate layer from the other layers.

4. Method for producing a fraction including antibacterial red blood cells, which comprises following steps;

(a)mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

(b)separating and collecting the upper layer from the other layers;

(c)treating the upper layer sample with hypotonic solution for a short period and then adding hypertonic solution into said upper layer sample to make isotonic solution; and

(d)adding incubated leucocytes into said isotonic solution.

5. Method for producing a fraction including bacterial proliferation inhibitory red blood cells comprising following steps;

(a)mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

(b) separating and collecting the intermediate layer from the other layers; and

(c) adding incubated leucocytes into said intermediate layer.

5       6. Antibacterial material included in the solution produced  
by following steps;

(a) mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

10 upper, intermediate, and lower layers;

(b) separating and collecting the upper layer from the other layers; and

(c)treating the upper layer sample with hypotonic solution for a short period and then adding hypertonic solution into said upper layer sample.

15 said upper layer sample.

7. Bacterial proliferation inhibitory material included in the solution produced by following steps;

(a) mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers; and

(b) separating and collecting the intermediate layer from the other layers.

8. Antibacterial material included in the solution produced  
25 by following steps;

(a) mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so

as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

(b) separating and collecting the upper layer from the other layers;

5 (c) treating the upper layer sample with hypotonic solution for a short period and then adding hypertonic solution into said upper layer sample to make isotonic solution; and

(d) adding incubated leucocytes into said isotonic solution.

10 9. Bacterial proliferation inhibitory material included in the solution produced by following steps;

(a) mixing human blood sample with dextran aqueous solution and maintaining said mixture stationarily for 60 to 75 min so as to fractionate this blood sample into three layers, the upper, intermediate, and lower layers;

15 (b) separating and collecting the intermediate layer from the other layers; and

(c) adding incubated leucocytes into said intermediate layer.



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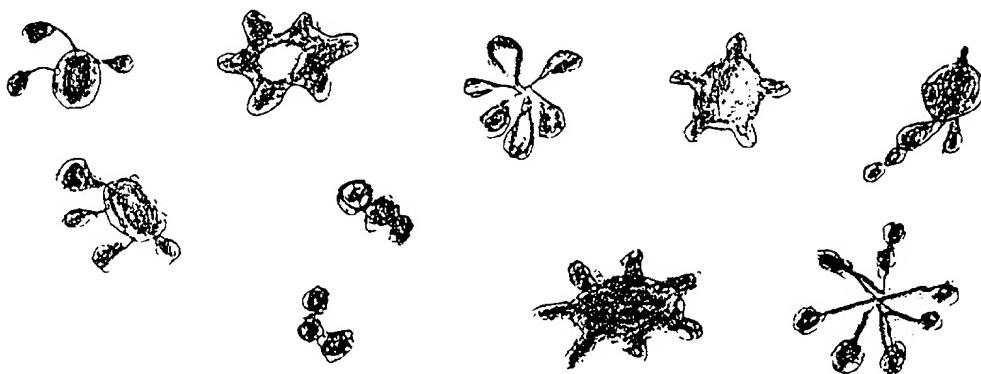
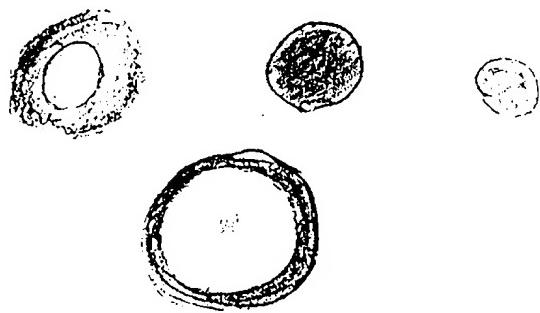
## ABSTRACT

METHOD FOR FRACTIONATING RED BLOOD CELLS AND ANTIBACTERIAL  
5 MATERIALS OR BACTERIAL PROLIFERATION INHIBITORS PRODUCED  
THEREBY

A method for fractionating red blood cells of human blood  
10 into three fractions comprises following steps; (a)human blood sample is mixed with dextran aqueous solution and maintained stationarily for 60 to 75 min to fractionate this blood sample into three layers; (b)the upper, intermediate, and lower layer samples are individually separated and collected; and (c)the  
15 upper layer sample is treated with hypotonic solution for a short period and then added with hypertonic solution. Further the invention provides antibacterial or bacterial proliferation inhibitory material produced by the method. This method can easily fractionate three different blood fractions  
20 including red blood cells as a main component and having different functions. These three different fractions can be applied to several clinical tests such as antibacterial test and the like.



02/20/97

**Fig.1****A Group****RC-derived cells (Masked Erythrocyte) = Old Erythrocyte****B Group****RC-derived cells (Masked Erythrocyte)****C Group****Non-Masked Erythrocyte(new erythrocyte)**

## DECLARATION FOR PATENT APPLICATION

(Foreign Agent Involved)

Docket Number (Optional)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

59227 U.S. PTO  
08803458

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention METHOD FOR FRACTIONATING RED BLOOD CELLS AND ANTI-BACTERIAL MATERIALS OR BACTERIAL PROLIFERATION INHIBITORS PRODUCED THEREBY, the specification 02720764 attached hereto unless the following box is checked.

was filed on \_\_\_\_\_ as United States Application Number or PCT International Application Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

## Prior Foreign Application(s)

No. 8-215552	JAPAN	August 15, 1996	Priority Claimed
(Number)	(Country)	(Day/Month/Year Filed)	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
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(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
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The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Takiro Kojima, P.A. as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: G.R. Myers, Reg. No. 24,897; T.P. Liniak, Reg. No. 33,415; J.W. Berenato, III, Reg. No. 30,546; and J.A. Rhoa, Reg. No. 37,515. Address all telephone calls to Joseph W. Berenato, III at telephone number (301) 365-8000. Address all correspondence to Myers, Liniak & Berenato 6550 Rock Spring Drive, Suite 240, Bethesda, Maryland 20817.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name) \_\_\_\_\_

Inventor's signature XXXXXXXXXXXXXX Date January 20, 1997

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Full name of second joint inventor, if any (given name, family name) \_\_\_\_\_

Second Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Additional inventors are being named on a separate sheet attached hereto.